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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of DONALD S. ANSON ET AL. Attorney Docket: 604-8

Serial Number: 06/839,215 Group Art Unit: 183

Filed: March 13, 1986 Examiner: J. Kushan

For: FACTOR IX PROTEIN

DECLARATION UNDER RULE 132

PROFESSOR GEORGE GOW BROWNLEE F.R.S. declares as follows:

1. I am the E.P. Abraham Professor of Chemical Pathology at the Sir William Dunn School of Pathology, University of Oxford, England and one of the inventors of the above-identified patent application. I have already made a Declaration, on 10th November 1988 and since then I have read the response dated 22nd December 1988 and the further office action from the United States Patent and Trademark Office.

2. The original papers (Suomela and Osterud et al.) while claiming apparent homogeneity, do not show clear evidence of this if the papers are examined critically. I have inspected an original print (not a photocopy) of the Suomela paper in European Journal of Biochemistry 71, 145-154 (1976) which has been cited. Such a print is normally available in a library. I refer first to Figure 2 which shows polyacrylamide gel electrophoresis of factor IX in the starting concentrate (track 1) and then after successive purifications (tracks 2-5). Figure 2, track 5 shows a higher molecular weight (MW) contaminant which is just visible. It is in the same position as the clearly visible contaminant in track 4 of the same figure. This

contaminant is not mentioned in the text by the authors, but is visible to my eyes. It is more obvious in the right hand half of lane 5 than in the left hand half of lane 5. Another fractionation of the same material is shown in Figure 3, track 4, where (arrowed) there are degradation products of lower MW and, equally clearly, but not arrowed, are higher MW contaminants. It should be noted that in the Discussion, Suomela points out that he has purified his material to apparent homogeneity (page 152 - my emphasis). This is the usual statement made by a cautious scientist, implying that there may still be traces of contamination observed, or indeed unobserved, because of the lack of sensitivity in the procedures.

3. From page 6 of the office action it seems that a misunderstanding has occurred concerning high molecular weight contamination of Suomela's product. In the response to the last office action it was pointed out that gel 4 of Figure 3 of Suomela showed high MW impurity "towards the top of the band". This should have read "towards the top of the gel". As the examiner will be well aware, in gel electrophoresis the higher MW proteins move more slowly in the gel and therefore accumulate towards the top, while the faster-moving lower molecular weight proteins appear in lower positions. The high molecular weight contaminant(s) mentioned by applicants appear above the band for factor IX and so cannot possibly be degradation products of lower molecular weight than factor IX. Consequently, the suggestions made on page 6 of the office action do not explain away the high molecular weight contaminant(s) shown in Suomela's Figures 2 and 3.

4. Turning to Osterud et al., J. Biol. Chem. 253, 5946-5951 (1978), I have also inspected an original print of this paper. In Figure 1, the authors ran a gel on 4 µg of factor IX, observing a single apparently homogenous material. However, in track C of Figure 1 I can see a high molecular weight band of MW about 68,000 (as well as two lower molecular weight bands at about 40,000 and 45,000). Moreover, they used a detection stain based on Coomassie blue which is sensitive only to about 0.1-0.2µg of protein. Clearly, therefore, they would be unable to observe a contaminant at less than the 2.5-5% level (0.2/4 x 100% = 5%), as this would be beyond the sensitivity of their methodology. Consequently, I regard Osterud et al.'s evidence of purity as extremely unconvincing. Also, since their procedure of purification is less thorough then Suomela's, I do not think that there is much of a case for supposing that Osterud et al.'s material could be pure, when Suomela's evidently is not. I note that Osterud et al. claim only "greater than 95% homogeneity" (page 5950, left-hand column, line 7).

5. No protein chemist believes a protein which he/she has purified ever reaches 100% purity. Let me illustrate this by reminding ourselves that

1 g mole is 6×10^{23} molecules (the Avogadro number)

$\therefore$ 60,000 g of factor IX (the MW approximately) = 6×10^{23} molecules

$\therefore$ 1 g = 10^{19} molecules

$\therefore$ 1 µg = 10^{13} molecules

Taking the Osterud et al. case, where 4 µg was loaded onto the gel (i.e. 4 x 10^{13} molecules), even if their material were 99% pure (which I doubt), the authors would have 1% of 4 x 10^{13} = 4 x 10^{11} molecules of contaminating proteins. Thus, it is theoretically virtually impossible, given the Avogadro number, to remove all contaminating protein molecules.

6. Present methods of detecting proteins on gels are improved on those used in the mid-70s and the method of DeMoreno et al. (Anal. Biochem. 151, 466-470, 1985) gives 10-100 times more sensitivity. Experience has shown that when this method is used, many preparations of protein, previously thought to be pure, clearly show evidence of contaminants.

7. I turn now to the issue of polymorphism of factor IX. I consider it to be beyond reasonable dispute that the polymorphism (to which reference was made in the last response) is an actual difference, at least in Caucasians. It can be shown to be so logically as follows:-

(i) The polymorphism Ala/Thr occurs in Caucasian populations with a frequency of 0.33 and 0.66 for the two alleles. Thus a male (XY) will have factor IX protein of one form or the other, depending on the allele encoded on his X chromosome. A female (XX) will either make one form or the other, or both if she is a heterzygote for this allele.

(ii) For the purposes of argument, if we assume we have a pool of plasma derived from 10 males, clearly it cannot be known without analysis whether each of these 10 males will be of the same allele (either the rarer or the commoner form). However, it is most likely, on probability grounds, they will be a mixture which will reflect the frequency of the alleles in the population at large. The probability then, in a pool of 10 males, that all 10 would produce factor IX derived solely from ~~one allele (or solely from the other allele) is~~ the commoner allele is $0.66^{10} = 1.56 \times 10^{-2}$ or that all 10 would produce factor IX derived from the rarer allele is $0.33^{10} = 1.53 \times 10^{-5}$ ~~the same as tossing a coin 10 times and it emerging 10 heads (or 10 tails) in a row. I work this out to be p = about 0.0003, or a chance~~ of ~~about 3 in~~ 1000. Clearly, put another way ~~p = 0.997~~ $p > 0.984$ that this pool of plasma of 10 males will produce a <u>mixture</u> of factor IX molecules. If the pool derives from a mixture of males and females (females being heterozygous and therefore increasing the chance of mixtures), p will be higher than ~~0.997~~ 0.984. If we consider pools of 100 or even 1,000 plasma samples, which are in practice used to produce commercial concentrates for patient treatment, then for all practical, sensible purposes we can be <u>certain</u> that the samples will be a mixture.

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8. The other fact which may not have been appreciated is that when a cDNA clone of factor IX is isolated, then as long as this clone derives from a single molecule of DNA entering a bacterium (and I am certain of this figure in relation to the factor IX cDNA clone), it doesn't matter whether this single entity derives from one liver or

not (although I believe that it is derived from a single male liver in the present case). The only condition whereby the Osterud et al. or Suomela preparations of factor IX would be monomorphic at this locus would arise:

(1) if the factor IX were produced from a single individual or a very low number of individuals; or (2) if the ethnic group for which the plasma was obtained were not Caucasian (the existence and extent of this polymorphism has not been studied extensively in other ethnic groups). It is impossible to say from the Osterud et al. and Suomela papers whether (1) and (2) applied, as no information is given on the exact number of donor plasma samples used or their origin. I would only comment that no one wishing to prepare purified factor IX from plasma, for commercial purposes, would ever think of using plasma from a single donor or even less than fifty donors, since the quantities of factor IX obtainable would be insignificant. Nor would anyone think of segregating Caucasian plasma from non-Caucasian plasma. The Suomela and Osterud et al. papers come from countries with a predominantly Caucasian population. It is reasonable to assume, therefore, that their plasma concentrates are predominantly of Caucasian origin. Thus, for all practical, sensible purposes, the polymorphism present in human plasma is a material difference which distinguishes factor IX produced by carrying out the Suomela or the Osterud et al. methods on a commercial scale from factor IX produced from a recombinant DNA present in a single clone (and not a mixture).

9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

1st July 1989	George G. Brownlee
Date	George Gow Brownlee